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EXHIBIT A

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Passive Immunity to Yersiniae Mediated by Anti-Recombinant V Antigen and Protein A-V Antigen Fusion Peptide

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LcrV (V antigen), a known unstable 37.3-kDa monomeric peptide encoded on the ca. 70-kb Lcr plasmid of *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*, has been implicated as a regulator of the low-calcium response, virulence factor, and protective antigen. In this study, *lcrV* of *Y. pestis* was cloned into protease-deficient *Escherichia coli* BL21. The resulting recombinant V antigen underwent marked degradation from the C-terminal end during purification, yielding major peptides of 36, 35, 34, and 32 to 29 kDa. Rabbit gamma globulin raised against this mixture of cleavage products provided significant protection against 10 minimum lethal doses of *Y. pestis* ($P < 0.01$) and *Y. pseudotuberculosis* ($P < 0.02$). To both stabilize V antigen and facilitate its purification, plasmid pPAV13 was constructed so as to encode a fusion of *lcrV* and the structural gene for protein A (i.e., all but the first 67 N-terminal amino acids of V antigen plus the signal sequence and immunoglobulin G-binding domains but not the cell wall-associated region of protein A). The resulting fusion peptide, termed PAV, could be purified to homogeneity in one step by immunoglobulin G affinity chromatography and was stable thereafter. Rabbit polyclonal gamma globulin directed against PAV provided excellent passive immunity against 10 minimum lethal doses of *Y. pestis* ($P < 0.005$) and *Y. pseudotuberculosis* ($P < 0.005$) but was ineffective against *Y. enterocolitica*. Protection failed after absorption with excess PAV, cloned whole V antigen, or a large (31.5-kDa) truncated derivative of the latter but was retained ($P < 0.005$) upon similar absorption with a smaller (19.3-kDa) truncated variant, indicating that at least one protective epitope resides internally between amino acids 168 and 275.

Established virulence factors of *Yersinia pestis*, the causative agent of bubonic plague, are encoded on the chromosome (e.g., iron transport functions and antigen 4), a ca. 100-kb toxin or Tox plasmid (murine exotoxin and capsular fraction 1 antigen), a ca. 70-kb low-calcium response or Lcr plasmid (yersinia outer membrane peptides termed Yops), and a ca. 10-kb pesticin or Pst plasmid (plasminogen activator). The enteropathogenic yersiniae (*Yersinia pseudotuberculosis* and *Yersinia enterocolitica*) possess only the Lcr plasmid and thus lack a number of determinants necessary for expression of severe systemic disease (4). The common Lcr plasmid mediates restriction of growth at 37°C unless the environment contains mammalian extracellular levels of Ca^{2+} (ca. 2.5 mM). Cells of *Y. pestis* arrested in this physiological state fail to synthesize stable RNA (13) or bulk vegetative protein (32, 33, 68) but either continue synthesis or induce expression of stress functions (e.g., GroEL-like protein) and most of the virulence factors noted above (Lcr$^+$) (32).

The Yops encoded by the Lcr plasmid are secreted peptides that, in *Y. pestis*, can be distinguished in vitro by whether they are released intact into culture supernatant fluids or appear as small fragments after undergoing posttranslational degradation (53, 54) mediated by the species-specific plasminogen activator (56). Export in vitro of degradable Yops in intact form by Pst plasmid-deficient enteropathogenic yersiniae is promoted by Lcr plasmid-encoded functions (35, 36) involving evident recognition without processing of the N-terminal end of these peptides (14, 34–37). Similarly, the stable (i.e., nondegradable) YopM of *Y. pestis* is secreted from the bacterium with an intact N terminus (48). Degradable Yops E (50, 51, 59, 62), H (2, 49, 59), K plus L (62), and probably B (22), as well as stable YopM (27, 28, 48), are established virulence factors. YpkA also belongs in this category (18). All of these degradable peptides possess properties consistent with roles as cytotoxins (18, 21, 22, 50), whereas YopM binds to thrombin and might thus function in concert with plasminogen activator during terminal disease (57) to promote hemorrhagic sequelae (27, 28, 48). Degradable YopD may serve to deliver cytotoxic Yops to target cells (22, 51), and stable YopN was assigned a role in sensing Ca^{2+} (17).

A putative virulence factor encoded by the Lcr plasmid (45) is LcrV (V antigen), initially described as a major exported peptide of wild-type *Y. pestis* (7, 9) and *Y. pseudotuberculosis* (11) and later identified in Lcr$^+$ isolates of *Y. enterocolitica* (12, 44). Results of genetic analysis positioned *lcrV* within an *lcrGVH-yopBD* operon (1, 22, 40, 45–47) and showed that a nonpolar deletion in *lcrV* promoted loss of the nutritional requirement for Ca^{2+} and resulted in avirulence (22, 46). These findings were interpreted as evidence indicating that V antigen serves as a regulator of the low-calcium response (1, 46). However, no mechanism of direct regulation has yet been defined for V antigen, and the possibility of a bifunctional role as a virulence factor remains plausible (46).

Evidence for the latter is primarily immunological and originated with the finding that rabbit antiserum raised against partially purified V antigen provided passive protection to mice against challenge by *Y. pestis* (26). This observation was later refined by demonstrating that both polyclonal antisera raised against highly purified V antigen (41, 66, 67) and a

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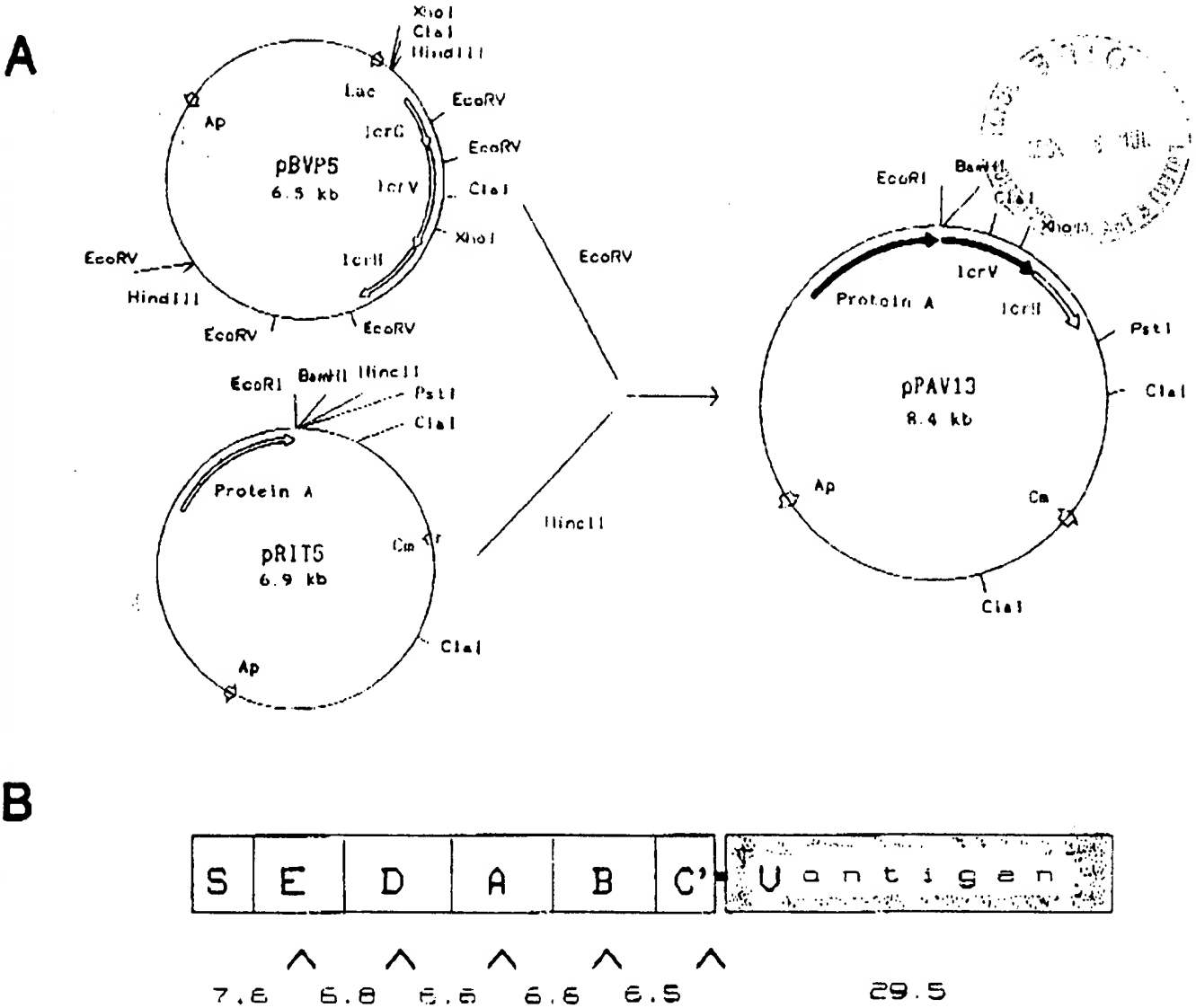

FIG. 1. Scheme of construction of recombinant plasmid pPAV13 encoding a staphylococcal protein A-V antigen fusion peptide termed PAV (A) and characterization of PAV (B). Sites of restriction endonuclease attack are indicated; Ap and Cm are locations of markers for resistance to ampicillin and chloramphenicol, respectively. Lac indicates the position of *lacZ*, which facilitates selection of recombinant plasmids in the vector pBluescript SK⁺. The genes *lcrG*, *lcrV*, and *lcrH* comprise a portion of the *lcrGVH-yopBD* operon of *Y. pseudotuberculosis* 995 (39), and the term protein A defines the location of the truncated protein A gene. Dark arrows in panel A represent the hybrid gene encoding PAV shown in panel B to consist of the signal sequence (S), IgG-binding domains (E to B), the defective domain C' that has lost the ability to bind IgG, and truncated V antigen that has lost the first 67 amino acids of its N-terminal end. Molecular masses in kilodaltons are indicated for each peptide arising after hydrolysis of the acid-labile Asp-Pro cleavage sites marked by arrowheads (65).

monoclonal anti-V antigen (41, 55) also provided passive immunity. Protection was attributed to neutralization by the antisera of an immunosuppressive activity mediated at least in part by V antigen (41) that prevented infiltration of host inflammatory cells to necrotic foci of infection in visceral organs (62, 67). The mechanism of this form of immunosuppression is presently unknown but probably involves inhibition of cytokine synthesis (41). Studies of immunosuppression and immunity mediated by V antigen have been seriously impeded by the penchant of this 37.3-kDa peptide to undergo evident autodegradation after the process of purification is initiated (6). In this report, we first show that recombinant V antigen expressed in protease-deficient *Escherichia coli* BL21 undergoes similar degradation. We next demonstrate that antisera raised against either this mixture of fragments or a stable staphylococcal protein A-V antigen fusion peptide (PAV) (which can be purified to homogeneity in one step by immunoglobulin G [IgG] affinity chromatography) can provide statistically significant protection against 10 minimum lethal doses of *Y. pestis* and *Y. pseudotuberculosis* but not *Y. enterocolitica*. Finally, we establish by absorption of anti-PAV with excess V antigen and progressively smaller truncated derivatives that passive immunity is mediated by at least one internal protective epitope.

TABLE 1. Purification of recombinant V antigen from a cell extract of *Escherichia coli* BL21(pKVE14)

Preparation	Vol (ml)	Amt of protein (mg/ml)	Total protein (mg)	Amt of V antigen (U/ml)[a]	Total V antigen (U)	Sp act[b]	% Recovery
Crude extract	200	26	5,200	280	56,000	11	100
Phenyl-Sepharose CL-4B	220	1.6	350	140	30,800	88	55
DEAE-cellulose	40	1.5	60	170	6,800	113	12.1
Sephacryl S-300SF	24	0.7	17	140	3,360	200	6.0
Ca hydroxylapatite	35	0.25	8.8	50	1,750	200	3.1
DEAE-cellulose (2nd separation)	18	0.1	1.8	15	270	150	0.5

[a] A unit of V antigen was defined as the reciprocal of the highest dilution capable of forming a visible precipitate against a standardized lot of rabbit polyclonal monospecific antiserum by diffusion in agar under conditions described previously (6, 26).
[b] Specific activity is in units per milligram of protein.

MATERIALS AND METHODS

Bacteria. *E. coli* K-12 XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F' *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]) (Stratagene, La Jolla, Calif.) was used as a host for genetic engineering manipulations, and protease-deficient *E. coli* BL21 (F$^-$ *ompT lon* r_B^- m_B^-) (Novagen, Madison, Wis.) was utilized for expression of *lcrGVH-yopBD* under control of the *tac* promoter and production of PAV and truncated protein A. *E. coli* BL21(DE3) was used for biosynthesis of cloned gene products under control of the T7 promoter (20). The latter strain is lysogenic for DE3 which carries the T7 RNA polymerase gene under control of *lacUV5* (63).

Passively immunized mice were challenged with wild-type cells of *Y. pseudotuberculosis* PB1/+ (11) or *Y. enterocolitica* WA of the highly virulent O:8 serotype (12). This purpose was accomplished with *Y. pestis* KIM by use of a nonpigmented mutant (23, 64) known to lack a spontaneously deletable ca. 100-kb chromosomal fragment encoding functions of iron transport and storage (16, 31); this isolate retained all other known chromosomally encoded virulence functions plus the Tox, Lcr, and Pst plasmids (15, 61). Mutants of this phenotype are virulent by intravenous injection (50% lethal dose, ca. 10 bacteria [66]) but not by peripheral routes of infection (50% lethal dose, $>10^7$ bacteria [5, 24]).

Plasmids. The vector pKK223-3 containing the *tac* promoter (Pharmacia, Uppsala, Sweden) was used to express a portion of the *lcrGVH-yopBD* operon of *Y. pestis* 358 (25) as described below. The vector pRIT5 (Pharmacia) encoding staphylococcal protein A was used for construction of gene fusions, as was the recombinant plasmid pBVP5 containing the *lcrGVH-yopBD* operon of *Y. pseudotuberculosis* (39). The latter was also used in preparation of deletion derivatives of *lcrV* yielding truncated derivatives of V antigen. The vector plasmid pBluescript SK$^+$ (Stratagene) was introduced into *E. coli* BL21(DE3) for use in absorption of antiserum.

DNA methods. Methods for preparation of plasmid DNA and its digestion with restriction enzymes, ligation, sequencing, and transformation into *E. coli* have been described previously (52). The 3.5-kb *Hin*dIII fragment of the Lcr plasmid of *Y. pestis* 358 was introduced into the expression vector pKK223-3. The resulting recombinant plasmid pKVE14 was then selected to ensure that the direction of transcription of the *lcrGVH* sequence corresponded to the action of the *tac* promoter.

The schema used to construct pPAV13 containing a hybrid gene encoding a portion of protein A of *Staphylococcus aureus* and V antigen of *Y. pseudotuberculosis* is shown in Fig. 1A. The 1.5-kb *Eco*RV fragment of recombinant plasmid pBVP5 (39) was introduced into the vector pRIT5 encoding truncated protein A. The latter, either alone or fused with V antigen, maintained its signal sequence and most IgG-binding domains but lost the region mediating association with the bacterial cell surface (42, 43) (Fig. 1B). As a consequence of this fusion, *lcrV* lost 201 bp, causing deletion of the first 67 amino acids comprising the N-terminal portion of V antigen. The resulting PAV thus contained 305 N-terminal amino acids from protein A and 259 C-terminal amino acids from V antigen (Fig. 1B).

Deletion variants of *lcrV* were constructed by reducing the size of the 3.5-kb *Hin*dIII fragment of pBVP5 to 2.2 kb by cleavage of the *Acc*I site downstream of *lcrH*. Prepared by this process, recombinant plasmid pBV513D contained the whole *lcrGVH* sequence under control of the T7 promoter (Fig. 2). Additional deletion variants were then prepared by digesting pBVP513D with exonuclease III followed by treatment with mung bean nuclease (52). The resulting set of plasmids retained the T7 promoter but lost progressively larger portions of the 3′ end of *lcrV* (which thus encoded a family of truncated V antigens that had lost correspondingly larger portions of the C-terminal end). Termini of each deletion variant were established by nucleotide sequencing and are shown in relationship to pPAV13 and pBVP5 with predicted molecular weights of the resulting truncated derivatives of V antigen (Fig. 2).

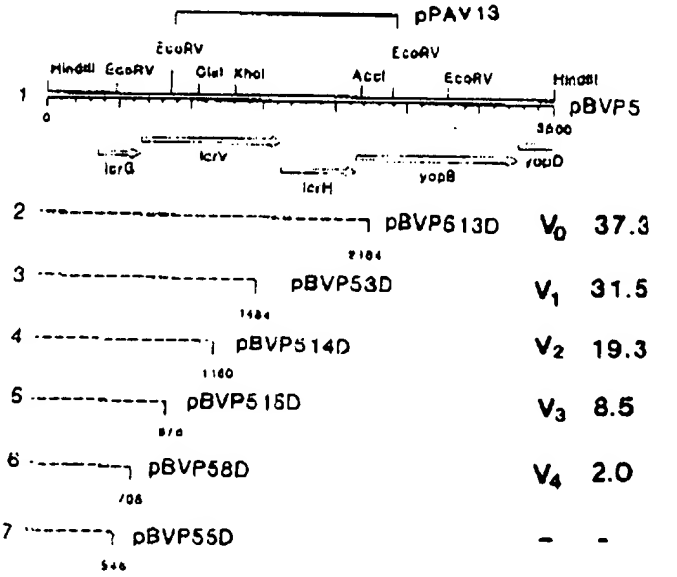

FIG. 2. Deletional variants constructed from pBVP5 consisting of the vector pBluescript SK$^+$ and the 3.5-kb *Hin*dIII fragment from the *lcrGVH-yopBD* operon of the Lcr plasmid of *Y. pseudotuberculosis* 995. The location of the *Eco*RV fragment used for construction of the fusion protein PAV is also indicated. Molecular masses (in kilodaltons) of truncated peptides of V antigen deduced from nucleotide sequences are shown on the right.

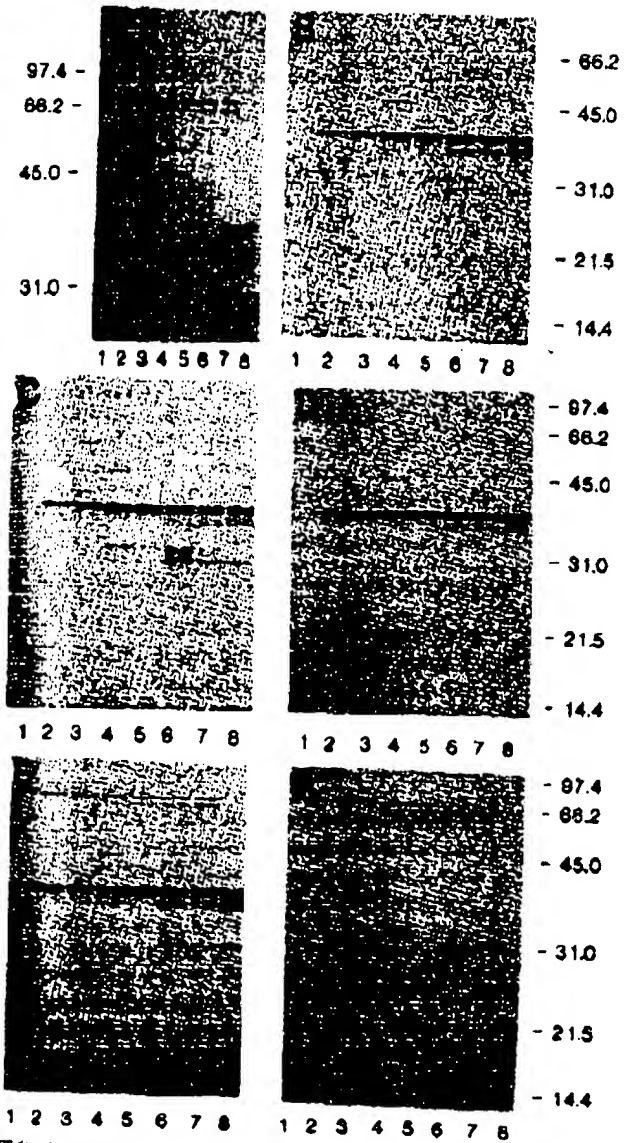

FIG. 3. (A) Silver-stained extended SDS–12.5% PAGE gel of whole cells of *E. coli* BL21 containing the vector plasmid pK223-3 (lane 1) or recombinant plasmid pKVE14 (lane 2). Whole cells of the latter were disrupted and centrifuged to prepare a cell extract (lane 3) that was fractionated by chromatography on phenyl-Sepharose CL-4B (lane 4), DEAE-cellulose (lane 5), Sephacryl S-300SF (lane 6), calcium hydroxylapatite (lane 7), and second-passage DEAE-cellulose (lane 8); V antigen appears as a major peptide of 37 kDa in lanes 2 through 8. The same samples were immunoblotted against rabbit polyclonal anti-native V antigen (B), mouse monoclonal anti-V antigen 15A4.8 (C), mouse monoclonal anti-V antigen 3A4.1 (D), rabbit polyclonal anti-PAV (E), and rabbit polyclonal anti-truncated staphylococcal protein A (F). Numbers on the left and right indicate molecular masses in kilodaltons.

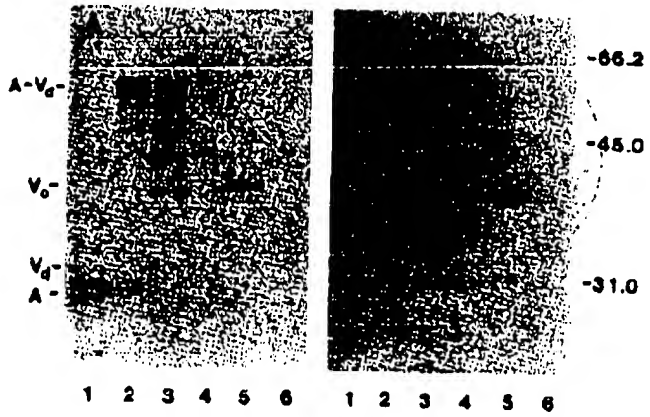

FIG. 4. Immunoblots prepared with polyclonal anti-native V antigen (A) or mouse monoclonal anti-V antigen 17A5.1 (B) directed against truncated protein A (lanes 1), PAV (lanes 2), PAV partially hydrolyzed by formic acid (lanes 3), PAV partially hydrolyzed by formic acid and then passed through the IgG-Sepharose 6FF column (lanes 4), whole Ca^{2+}-starved Lcr$^+$ cells of *Y. pestis* KIM (lanes 5), and whole Ca^{2+}-starved Lcr$^-$ cells of *Y. pestis* KIM (lanes 6); A-V_d, V_o, V_d, and A indicate the positions of PAV, native V antigen (37.3 kDa), truncated V antigen (29.5 kDa), and truncated protein A, respectively. Human gamma globulin was used to block nonspecific reactions of monoclonal antibodies against IgG-binding domains of protein A (29). Numbers on the right indicate molecular masses in kilodaltons.

Termination of translation of these truncated derivatives of V antigen occurs in the vector portion of the established sequence of pBluescript SK$^+$ (Stratagene) at bp 726 (frame 1) for pBVP53D, pBVP515D, and pBVP58D, thus increasing V_1, V_3, and V_4 by 9 amino acids; termination occurs at bp 776 (frame 3) for pBVP514D, thereby increasing V_2 by 25 amino acids.

Purification of recombinant V antigen. Cells of *E. coli* BL21(pKVE14) were grown in fermentors as described previously (6) in medium consisting of 3% Sheffield NZ Amine, Type A (Kraft, Inc., Memphis, Tenn.), 0.5% NaCl, 1% lactose, and ampicillin (100 μg/ml) at 37°C and harvested by centrifugation (10,000 × *g* for 15 min) upon achieving an optical density at 620 nm of about 1.2. After disruption in a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) and removal of insoluble matter by centrifugation (10,000 × *g* for 30 min), V antigen was subjected to purification by an established procedure involving use of hydrophobic interaction chromatography with phenyl-Sepharose CL-4B (Pharmacia), ion-exchange chromatography with DEAE-cellulose (Whatman Inc., Clifton, N.J.), gel filtration with Sephacryl S-300F (Pharmacia), and calcium hydroxylapatite chromatography on Bio-Gel HTP (Bio-Rad, Richmond, Calif.) (6). The original process was supplemented by a second chromatographic separation on DEAE-cellulose (linear gradient from 0 to 0.35 M NaCl) in order to remove high-molecular-weight material unique to *E. coli*.

Preparation of truncated protein A and PAV. Cells of *E. coli* carrying pPAV13 or pRIT5 were grown to late log phase at 37°C in Luria broth containing ampicillin (50 μg/ml). Purification of these recombinant proteins was accomplished by affinity chromatography on IgG-Sepharose 6FF (Pharmacia) according to directions supplied by the manufacturer. Briefly, this process involved harvesting the organisms by centrifugation (10,000 × *g* for 15 min) with resuspension at a ca. 10-fold increase in number in 0.01 M Tris-HCl, pH 8.0 (column buffer). Lysis was accomplished by initial addition of lysozyme (5 mg/ml) and then, after incubation for 1 h, further addition of Triton X-100 (0.1%), whereupon incubation was continued for 3 to 4 h. After clarification by centrifugation (10,000 × *g* for 30 min), samples of 400 ml of the resulting soluble proteins were passed through a column (10 by 100 mm) containing a 10-ml packed volume of affinity resin that selectively bound truncated protein A or PAV. After addition and elution of 10 void volumes of column buffer to remove contaminating

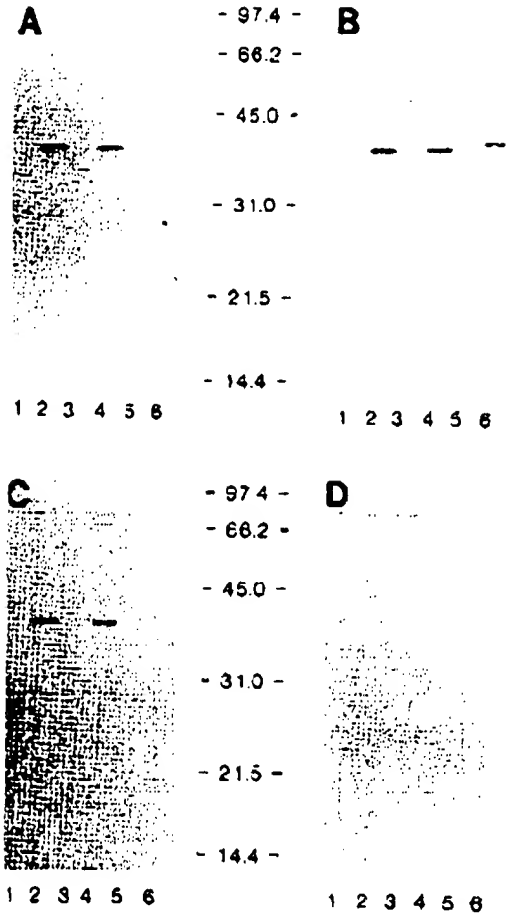

FIG. 5. Immunoblots prepared with absorbed rabbit polyclonal anti-native V antigen purified from *Y. pestis* KIM (A), anti-recombinant V antigen (B), anti-PAV (C), and anti-truncated protein A (D) directed against Ca^{2+}-starved whole cells of Lcr⁻ *Y. pestis* KIM (lanes 1), Lcr⁺ *Y. pestis* KIM (lanes 2), Lcr⁺ *Y. pseudotuberculosis* PB1 (lanes 3), Lcr⁺ *Y. pseudotuberculosis* PB1 (lanes 4), Lcr⁻ *Y. enterocolitica* WA (lanes 5), and Lcr⁺ *Y. enterocolitica* WA (lanes 6). Numbers down the middle indicate molecular masses in kilodaltons.

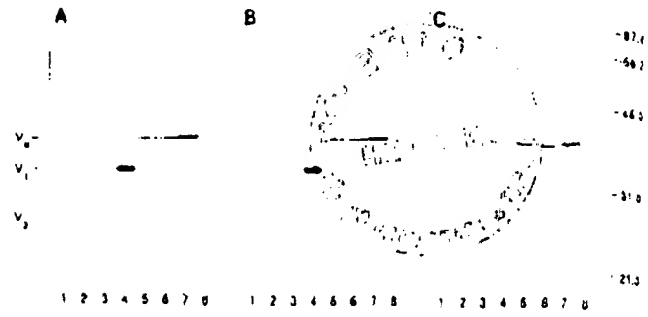

FIG. 6. Immunoblots prepared with absorbed rabbit polyclonal anti-native V antigen (A), mouse monoclonal anti-V antigen 15A4.8 (B), and mouse monoclonal anti-V antigen 17A5.1 (C) against extracts of *E. coli* BL21(DE3)/pBluescript SK⁺ (lanes 1), *E. coli* BL21(DE3)/pBVP515D (lanes 2), *E. coli* BL21(DE3)/pBVP514D (lanes 3), *E. coli* BL21(DE3)/pBVP513D (lanes 4), *E. coli* BL21(DE3)/pBVP513D (lanes 5), *E. coli* BL21(DE3)/pBVP5 (lanes 6), Lcr⁺ *Y. pestis* KIM (lanes 7), and Lcr⁻ *Y. pestis* KIM (lanes 8). Numbers on the right indicate molecular masses in kilodaltons.

matter, the recombinant proteins were eluted with 0.2 M acetic acid (ca. pH 3.4), immediately frozen, and lyophilized. The resulting purified truncated protein A and PAV were then used directly for qualitative analysis and immunization.

Acid hydrolysis of PAV. Purified PAV was treated with 70% formic acid for 20 h at 30°C to cleave the four labile Asp-Pro peptide bonds within the truncated protein A domain (65) and the additional site located at the junction with V antigen (42) (Fig. 1B). After dialysis against column buffer, the partial hydrolysate was again passed through the IgG-Sepharose 6FF column as described above. In this case, the V antigen moiety plus fragments of the protein A domain lacking IgG-binding sites were immediately eluted whereas residual unhydrolyzed PAV remained bound to the affinity resin.

Preparation of truncated derivatives of V antigen. Cells of *E. coli* BL21(DE3) transformed with pBVP5 and its deleted variants as well as the negative control pBluescript SK⁺ were grown in fermentors, harvested, and disrupted as described previously. After removal of insoluble material by centrifugation (10,000 × *g* for 30 min), the resulting concentrated cell extract was subjected to molecular sieving on a column (5 cm by 1.5 m) of Sephadex G100 (Pharmacia) in 0.05 M CHES [2-(*N*-cyclohexylamino)-ethanesulfonic acid] buffer, pH 9.0. Samples containing V antigen or its truncated derivatives were identified by silver staining or immunoblotting, pooled, dialyzed against 0.05 M Tris-HCl, pH 8.0, and applied to a column (2.5 by 46 cm) of DEAE-cellulose equilibrated in the same buffer. All forms of V antigen became absorbed during this process, and, after passage of 2 void volumes of column buffer, they were eluted by batchwise application of the buffer containing 0.5 M NaCl. After dialysis, these concentrated samples were used directly to absorb IgG isolated from a known protective antiserum raised against PAV (described below) in order to determine the location of protective epitopes.

Preparation of antisera. Rabbit polyclonal antiserum raised against V antigen purified from *Y. pestis* KIM, termed anti-native V antigen, has been characterized previously (41) and was used as a positive immunological control. This antiserum plus the two rabbit polyclonal antisera directed against highly purified truncated protein A or PAV were obtained by use of Freund's adjuvant as described previously (66). Less toxic TiterMax (Hunter's TiterMax no. R-1; CytRx Corp., Norcross, Ga.) was used to immunize rabbits against V antigen prepared from *E. coli* BL21(pKVE14); this antiserum was termed anti-recombinant V antigen. Neither the latter nor antisera raised against the fusion proteins were absorbed with material from Lcr⁻ bacteria, although highly purified gamma globulin was isolated from these reagents by the procedure used previously (66).

Methods used for the preparation of monoclonal antibodies recognizing nonconformational epitopes of V antigen have been described previously (3). As illustrated below, the first group of these antibodies reacted with an epitope present on the last 50 amino acids comprising the C-terminal part of V antigen (amino acids 276 to 326), as judged by ability to recognize V_6 (whole V antigen) but not V_1 or V_2 (monoclonal antibodies 3A4.1, 17A5.1, and 17A4.6). In contrast, monoclonal antibody 15A4.8 reacted with V_6 and V_1 but not V_2, indicating affinity for a shared internal epitope (amino acids 168 to 275).

Selective absorption of anti-PAV. Highly purified IgG prepared from anti-PAV was treated with excess PAV, V_6, or its truncated derivative V_1 or V_2 according to an established protocol (66). This process involved gentle aeration of the

TABLE 2. Ability of IgG isolated from normal rabbit serum and from antisera raised against native V antigen, recombinant V antigen, PAV, and truncated protein A to provide passive immunity against intravenous challenge with 10 minimum lethal doses of *Yersiniae*[a]

Challenge organism	Source of IgG[a]	No. of mice surviving on day after infection																No. dead/ total no.	P[b]
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	21		
Y. pestis KIM	Normal serum	10	10	10	10	6	4	1	0									10/10	
	Anti-native V antigen	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	0/10	<0.005
	Anti-recombinant V antigen	10	10	10	10	8	6	6	6	6	6	6	6	6	6	6	6	4/10	<0.05
	Anti-PAV	10	10	10	10	10	10	10	10	10	9	9	9	9	9	9	9	1/10	<0.01
	Anti-truncated protein A	10	10	10	10	3	1	0										10/10	NS
Y. pseudotuberculosis PB1	Normal serum	10	10	10	8	8	4	1	0									10/10	
	Anti-native V antigen	10	10	10	10	9	8	7	7	7	4	4	4	4	4	4	4	6/10	<0.05
	Anti-recombinant V antigen	10	10	10	8	8	8	8	8	8	7	5	5	5	5	5	5	5/10	<0.02
	Anti-PAV	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	0/10	<0.005
	Anti-truncated protein A	10	10	10	8	2	0											10/10	NS
Y. enterocolitica WA	Normal serum	10	10	10	10	10	10	6	3	2	0							10/10	
	Anti-native V antigen	10	10	10	10	10	10	8	6	4	2	1	1	1	1	1	1	9/10	NS
	Anti-recombinant V antigen	10	10	10	10	10	10	10	7	7	4	3	3	3	3	3	3	7/10	NS
	Anti-PAV	10	10	10	10	10	10	9	7	4	2	0						10/10	NS
	Anti-truncated protein A	10	10	10	10	10	10	7	4	2	0							10/10	NS

[a] Mice received 100 μg of IgG in 0.1 ml of 0.033 M $KHPO_4$, pH 7.0, by intravenous injection on postinfection days 1, 3, and 5 (except for anti-recombinant V antigen, of which 500 μg was injected).

[b] Determined by Fisher's exact probability test; NS, not significant.

solution of IgG with an excess of a given antigen for 30 min at 37°C and then overnight incubation at 4°C. Precipitated material was removed by centrifugation (10,000 × *g* for 30 min), and then the same process of absorption was repeated twice. Remaining free IgG and putative small IgG-V antigen complexes were then precipitated by 50% saturated $(NH_4)_2SO_4$, dialyzed against 0.05 M Tris-HCl, pH 7.8, and purified on a column (1.5 by 30 cm) of DEAE-cellulose by elution with the same buffer according to the method initially used for isolation of IgG. All forms of free V antigen or any IgG-V antigen complexes remaining after absorption were removed by this process. As a consequence, a set of highly specific antisera that progressively lost the ability to recognize the epitopes shared by truncated derivatives of V antigen were prepared.

Immunoblotting. Alkaline phosphatase conjugated with anti-rabbit or anti-mouse IgG (Sigma Chemical Co., St. Louis, Mo.) was usually used as a secondary antibody during immunoblotting by procedures described previously (53, 54). These protocols were designed, in analysis of purified fractions, to maintain constant total activity of native V antigen (ca. 0.1 U per lane) and, in all other determinations, to maintain constant protein levels (7 to 10 μg per lane for cell lysates and 0.5 μg per lane for pure proteins). To prevent nonspecific reactions of monoclonal antibodies with truncated protein A and its derivatives, the nitrocellulose filter was first blocked with 5% fetal calf serum as usual and then incubated overnight with 1% normal human gamma globulin (Calbiochem, San Diego, Calif.); the latter (0.5%) was also added to solutions of primary and secondary antibodies (29). Fc-specific anti-mouse IgG (A-1418; Sigma) was used as a secondary antibody during immunoblotting of fusion proteins and their derivatives with monoclonal antibodies.

Passive immunity. The ability of the antisera and preparations of purified IgG described above to provide passive immunity in Swiss Webster mice was determined by defined methods (41, 66). This procedure involved intravenous injection of 10 minimum lethal doses of *Y. pestis* (10^2 bacteria), *Y. pseudotuberculosis* (10^2 bacteria), or *Y. enterocolitica* (10^3 bacteria) followed by intravenous administration of either 100 or 500 μg of purified IgG on postinfection days 1, 3, and 5.

Miscellaneous. Peptides were located in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, prepared as previously described (53, 54), by silver staining (38). Soluble protein was determined by the method of Lowry et al. (30). The statistical significance of the observed ability to provide passive immunity was determined by use of Fisher's exact probability test.

RESULTS

Degradation of recombinant V antigen. Recombinant plasmid pKVE14 containing the *lcrGVH-yopBD* operon of *Y. pestis* under control of the *tac* promoter was transferred into protease-deficient *E. coli* BL21. After growth in fermentors, the bacteria were disrupted and the resulting extract was used to prepare nearly homogeneous recombinant V antigen (Table 1) by a method established for Ca^{2+}-starved cells of *Y. pestis* (6). An additional step involving a second separation with DEAE-cellulose was necessary to eliminate major higher-molecular-weight proteins present in cytoplasm of *E. coli* BL21.

The initial specific activity of recombinant V antigen was almost fivefold greater than that obtained from *Y. pestis* starved of Ca^{2+} (6). Nevertheless, significant loss of precipitin activity occurred during every step of purification (Table 1). This phenomenon, as judged by inspection of a silver-stained lane gel (Fig. 3A), reflected gradual loss of the native 37-kDa form of V antigen with emergence of ca. 36- and 32-kDa and possibly smaller degradation forms. Analysis by immunoblotting was undertaken to prove that these new peptides shared epitopes with and thus arose from native V antigen. Use of rabbit polyclonal anti-native V antigen (Fig. 3B) or mouse monoclonal antibody 15A4.8, directed against a centrally located epitope (Fig. 3C), demonstrated emergence of new ca. 36-, 35-, and 34-kDa products early during the course of purification, with later appearance of a series of smaller fragments ranging from 32 to 29 kDa. The latter were not recognized by mouse monoclonal antibody 3A4.1 directed against an epitope located near the C-terminal end of native V antigen (Fig. 3D). These findings indicate that recombinant V antigen produced in the cytoplasmic background of *E. coli*

FIG. 7. Immunoblots prepared with rabbit polyclonal anti-PAV without absorption (A) and after exhaustive absorption with preparations of *E. coli* BL21(DE3) transformed with pBluescript SK$^+$, pVBPS14D, pBVPS3D, or pBVPS containing shared proteins alone (B), shared proteins plus excess V_2 (C), shared proteins plus excess V_1 (D), or shared proteins plus excess V_0 (E), respectively. Extracts of *E. coli* BL21(DE3)/pBVPS containing V_0 (lanes 1), *E. coli* BL21(DE3)/pBVPS3D containing V_1 (lanes 2), *E. coli* BL21(DE3)/pBVPS14D containing V_2 (lanes 3), and *E. coli* BL21(DE3)/pBluescript SK$^+$ (vector plasmid control) (lanes 4) are shown. Numbers on the right indicate molecular masses in kilodaltons.

BL21 undergoes evident spontaneous hydrolysis in a manner similar to that observed for native V antigen expressed in *Y. pestis* (6) and that this process of degradation is initiated at the C-terminal end of the peptide.

Characterization of truncated protein A and PAV. Additional constructions encoding a portion of the structural gene for staphylococcal protein A alone or this gene fused with *lcrV* (Fig. 1A) were found, after transformation into *E. coli* BL21, to promote significant synthesis of truncated protein A and PAV, respectively, as judged by the intensity and specificity of reactions observed in the immunoblots described below. These two peptides were purified to homogeneity in one step by affinity chromatography and then analyzed by immunoblotting. Polyclonal anti-native V antigen reacted nonspecifically with truncated protein A (Fig. 4A, lane 1) and both specifically and nonspecifically with PAV (Fig. 4A, lane 2). Proof that polyclonal anti-V antigen specifically recognized PAV and its derivatives (shown in lanes 2, 3, and 4 of Fig. 4A) was obtained by blocking the protein A domain with human gamma globulin and then immunoblotting with a monoclonal anti-V antigen. This process prevented visualization of truncated protein A (Fig. 4B, lane 1). Accordingly, all of the remaining bands visible in Fig. 4B reflect the occurrence of a specific interaction with an epitope of V antigen. Multiple bands appearing in samples of both truncated protein A (Fig. 4A, lane 1) and PAV (Fig. 4A, lane 2) represent accumulation of native and degraded forms of the protein A domain (Fig. 1B) within the periplasm of *E. coli* BL21 (19). To prove that the linked V antigen domain was stable, a sample of PAV was hydrolyzed with 70% formic acid to cleave acid-labile Asp-Pro linkers (Fig. 1B), neutralized, and then applied to the affinity column. Essentially pure truncated V antigen (V_d) emerged immediately (Fig. 4B, lane 4). The absence of multiple bands in this sample indicates that the V antigen domain within PAV (Fig. 1B) did not undergo degradation during purification as was described above to occur with free V antigen.

The number of total units of PAV purified by affinity chromatography was always identical to that present in the crude extract applied to the column. No significant loss of purified PAV occurred during storage in 0.01 M Tris-HCl (pH 7.8) for 1 week at 4°C.

Characterization of antisera raised against recombinant V antigens. Preparations of homogeneous gamma globulin were isolated from unabsorbed rabbit antisera raised against purified recombinant V antigen, PAV, and truncated protein A. The specific reaction obtained by immunoblotting Lcr$^+$ and Lcr$^-$ yersiniae containing the native 37-kDa V antigen of *Y. pestis* and *Y. pseudotuberculosis* and the 42-kDa V antigen of *Y. enterocolitica* (40) with control absorbed anti-native V antigen (Fig. 5A) was duplicated with anti-recombinant V antigen (Fig. 5B) and anti-PAV (Fig. 5C) but not with anti-truncated protein A (Fig. 5D). However, normal serum (data not illustrated), as well as the three unabsorbed antisera, also recognized unknown high-molecular-mass antigens (ca. 70 kDa) shared by Lcr$^+$ and Lcr$^-$ organisms. Anti-PAV (Fig. 3E) but not anti-truncated protein A (Fig. 3F) reacted with the same degradation products of recombinant V antigen that were identified upon assay with anti-native V antigen (Fig. 3B) and monoclonal antibody 15A4.8 (Fig. 3C).

Passive immunity mediated by anti-recombinant V antigens. As anticipated from prior work (66), control anti-native V antigen provided significant passive immunity against intravenous challenge with 10 minimum lethal doses of Lcr$^+$ cells of *Y. pestis* ($P < 0.005$) and *Y. pseudotuberculosis* ($P < 0.05$) but not *Y. enterocolitica* (Table 2). Anti-recombinant V antigen provided similar protection against challenge with *Y. pestis* ($P < 0.01$) and *Y. pseudotuberculosis* ($P < 0.02$), as did anti-PAV ($P < 0.01$ for *Y. pestis* and $P < 0.005$ for *Y. pseudotuberculosis*), whereas treatment with anti-truncated protein A was without effect.

Truncated V antigens. A series of recombinant plasmids containing deletions of increasing size in *lcrV* of *Y. pseudotuberculosis* was constructed; predicted molecular weights of the resulting entire V antigen (V_0) and its truncated derivatives (V_1 to V_4) are given in Fig. 2. The expression and actual sizes of these peptides were determined by immunoblotting. V_0 and V_1 exhibited strong reactions against anti-native V antigen, which barely detected V_2 (Fig. 6A); no interaction with V_3 or V_4 was observed (data not illustrated). Monoclonal antibody 15A4.8 failed to react with V_2 but recognized both V_0 and V_1 (Fig. 6B), indicating that its target epitope resides internally within the primary structure shared between the C-terminal ends of V_2 and V_1 (amino acids 168 to 275). In contrast, monoclonal antibody 17A5.1 recognized only V_0 (Fig. 6C), demonstrating that its target epitope resides within the amino acid sequence located between the C-terminal ends of V_1 and V_0 (amino acids 276 to 326). Identical results were obtained with mouse monoclonal antibodies 3A4.1 and 17A4.6 (data not illustrated). These results demonstrate that V_0 and V_1 were produced in abundance. Significant levels of less antigenic V_2 (as opposed to V_3 and V_4) were also expressed, as judged by the ability of this peptide to selectively remove specific antibodies from anti-PAV (described below). These results suggest that polyclonal antisera directed against V antigen primarily recognize epitopes located near the C-terminal rather than the N-terminal end of the peptide.

Selective absorption of anti-PAV. Cells of *E. coli* BL21 (DE3) carrying plasmids pBVP513D, pBVP53D, and pBVP 514D encoding V_0, V_1, and V_2, respectively, were induced in fermentors, and, after disruption, the resulting cytoplasmic extracts were subjected to a process involving separation by size and net charge that resulted in isolation of sufficient concentrations of the three peptides to permit selective absorption of anti-PAV. As shown in Fig. 7A, unabsorbed anti-PAV recognized V_0, V_1, and V_2, as well as the high-molecular-weight antigens noted previously (Fig. 3E and F and 5C and D). Antibodies to the latter could be removed by absorption with excess product obtained by parallel purification from extracts of control cells of *E. coli* BL21(DE3) containing the vector pBluescript SK$^-$ alone (Fig. 7B). Similar

TABLE 3. Ability of IgG isolated from rabbit polyclonal anti-PAV to provide passive immunity against intravenous challenge with 10 minimum lethal doses of *Y. pestis* KIM following absorption with excess PAV, V antigen, and truncated derivatives V_2 and V_1

Source of IgG[a]	Product used for absorption	*Y. pestis* KIM: No. of mice surviving on day after infection: 0	1	2	3	4	5	6	7	14	21	No. dead/total no.	*P*[b]	*Y. pseudotuberculosis* PB1: No. of mice surviving on day after infection: 0	1	2	3	4	5	6	7	14	21	No. dead/total no.	*P*[b]
None	None	5	5	5	5	3	3	0				5/5		5	5	5	5	4	3	0				5/5	
Normal serum	None	5	5	5	5	4	3	0				5/5	NS	5	5	5	5	4	3	0				5/5	NS
Anti-truncated protein A	None	5	5	5	5	4	3	1	0			5/5	NS	5	5	5	5	3	4	1	0			5/5	NS
Anti-PAV	None	5	5	5	5	5	5	5	5	5	5	0/5	<0.005	5	5	5	5	5	5	5	5	5	5	0/5	<0.005
Anti-PAV	Vector	5	5	5	5	5	5	5	5	5	5	0/5	<0.005	5	5	5	5	5	5	5	5	5	5	0/5	<0.005
Anti-PAV	V_2	5	5	5	5	5	5	5	5	5	5	0/5	<0.005	5	5	5	5	5	5	5	5	5	5	0/5	<0.005
Anti-PAV	V_1	5	5	5	5	5	4	3	0			5/5	NS	5	5	5	5	2	0					5/5	NS
Anti-PAV	V_0	5	5	5	5	4	3	1	0			5/5	NS	5	5	5	5	4	2	1	0			5/5	NS
Anti-PAV	PAV	5	5	5	5	4	3	0				5/5	NS	5	5	5	5	4	3	0				5/5	NS

[a] Mice received 100 μg in 0.1 mg of 0.033 M KH_2PO_4, pH 7.0, by intravenous injection on postinfection days 1, 3, and 5.
[b] Determined by Fisher's exact probability test. NS, not significant.

absorption with excess products prepared from isolates of *E. coli* BL21(DE3) carrying pBVP514D, pBVP53D, and pBVP5 progressively removed antibodies directed specifically against V_2 (Fig. 7C), V_1 (Fig. 7D), and V_0 (Fig. 7E), respectively.

Passive immunity mediated by anti-PAV absorbed with excess truncated V antigens. IgG purified from anti-PAV, absorbed as described above with excess V_0 or its truncated derivatives, was used to assay for ability to provide passive immunity against 10 minimal lethal doses of Ler$^+$ cells of *Y. pestis* or *Y. pseudotuberculosis*. In this determination (Table 3), lethality to untreated mice was absolute and occurred rapidly in a pattern similar to those observed for controls treated with purified normal IgG or IgG isolated from anti-truncated protein A. In contrast, all mice survived following administration of IgG from unabsorbed anti-PAV ($P < 0.005$) or that from anti-PAV absorbed with excess preparation of vector ($P < 0.005$) or V_2 ($P < 0.005$). Similar absorption of IgG from anti-PAV with excess V_1, V_0, or PAV itself rendered the antiserum ineffective. This finding provides formal proof that V antigen per se is a protective antigen and indicates that at least one epitope responsible for immunity resides internally within the primary structure spanning the C-terminal end points of V_2 and V_1 (amino acids 168 to 275).

DISCUSSION

Experimental evidence supporting the assumption that anti-V antigen provides immunity against plague was initially limited to the findings that active immunization with V antigen-rich fractions (10) or passive immunization with antisera raised against such fractions (26) promoted protection against experimental disease. Later concerns that additional Ler$^+$-specific antigens may have contributed to this immune state were minimized upon use of modern preparative methods that permitted recovery of nearly homogeneous V antigen (6). Nevertheless, antisera raised against lots of V antigen purified by use of these procedures often contained antibodies directed against highly antigenic contaminating proteins, especially Yops, present at trace levels in the final product used for immunization. Although these antibodies could easily be removed by absorption with outer membranes of Ler$^-$ yersiniae, this process necessitated introduction of potential immunomodulators, including lipopolysaccharide, known to possess the capability of stimulating nonspecific resistance to infection. Occurrence of this possibility was minimized by discovery of a protective monoclonal antibody (41, 55) and by purifying the gamma globulin from absorbed polyclonal antisera prior to injection (41, 66).

Remaining concerns that these precautions were insufficient to ensure monospecificity of polyclonal antisera were largely eliminated in the present study by use of gamma globulin raised against highly purified V antigen cloned in *E. coli*. Nevertheless, the occurrence of continuous degradation throughout the course of purification permitting only a fraction of the final product to exist as native V antigen also rendered this process unsatisfactory. The resulting low yield was insufficient to allow widespread immunization, although gamma globulin purified from antiserum raised against the final mixture of immunogenic peptides provided satisfactory immunity against *Y. pestis* and *Y. pseudotuberculosis*. The observation that cloned V antigen expressed in the protease-deficient background of *E. coli* BL21, like native V antigen purified from *Y. pestis*, underwent marked degradation during preparation is consistent with either the occurrence of autocatalytic hydrolysis or conversion to a steric form after partial purification, resulting in vulnerability to the inherent stresses of physical isolation (or to distinct contaminating proteases).

These problems concerning specificity and degradation were resolved upon development of the stable fusion protein PAV that could be isolated in one step at high yield in a homogeneous state. Rabbit polyclonal anti-PAV, like anti-native or anti-recombinant V antigen, was effective in providing passive immunity against *Y. pestis* and *Y. pseudotuberculosis*. This finding emphasizes that expression of protection did not require the presence of antibody against LcrG (linked upstream) or N-terminal epitopes of V antigen, because these sequences were absent in PAV used for immunization. The decision to sacrifice the N-terminal rather than the C-terminal end of V antigen to construct a fusion with protein A was based in part on the assumption that this region, like the N termini of Yops noted above, is involved in an exit reaction rather than catalysis of some biological activity directed against the host. Absorption of anti-PAV with excess truncated derivatives of V antigen lacking LcrH (linked downstream) provided further information about the location of protective epitopes. In these experiments, sufficient PAV, V_0, V_1, V_2, or a parallel preparation from cells carrying the plasmid vector alone was added to anti-PAV to selectively remove all corresponding antibodies detectable by immunoblotting. Assay of the resulting antisera showed that absorption with excess PAV, V_0, or V_1 but not V_2 or the vector control removed all protective antibodies. This

observation suggests that at least one protective epitope is located between the C-terminal points of V_2 and V_1 (amino acids 168 to 275). Demonstration that a monospecific antiserum loses its ability to provide passive immunity upon absorption with an excess of its opposing antigen provides formal proof that that antigen is protective. This criterion was met for V antigen in this study.

As already noted, considerable evidence that V antigen is exported into the external environment without transient accumulation at the bacterial surface has accrued (8, 26, 58, 60). This phenomenon argues against the possibility that anti-V antigen functions as an opsonin. The peptide may thus serve as a soluble mediator of disease. It is an axiom that the maximum increase in 50% lethal dose that can be provided by antibody directed against a given soluble virulence factor cannot exceed the increase in 50% lethal dose observed to occur in nonimmunized controls challenged with a mutant lacking the same virulence factor. Mutational loss of V antigen in *Y. pestis* results in at least a 10^6-fold decrease in lethality (46). Full active immunization of mice with PAV may result in an equivalent increase in 50% lethal dose following challenge with Lcr^+ cells of *Y. pestis*. In contrast, anti-V antigen was clearly ineffective in providing passive immunity against infection by highly invasive serotype O:8 cells of *Y. enterocolitica*. We are presently attempting to determine the explanation for this interesting distinction.

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